DESCRIPTION

CYTOLETHAL DISTENDING TOXINS AND DETECTION OF CAMPYLOBACTER BACTERIA USING THE SAME AS A TARGET

Cross References

This application is a 371 National Phase application of International Patent Application Serial No. PCT/JP2004/018042, filed December 3, 2004, which claims priority to Japanese Patent Application No. 2003-408103, filed December 5, 2003, which are incorporated herein by reference in their entirety noting that the current application controls to the extent there is any contradiction with any earlier applications and to which applications we claim priority under 35 USC §120 and 119.

Technical Field

The present invention relates to the cytolethal distending toxins of *Campylobacter coli* and polynucleotides encoding the same. The present invention also relates to methods for determining the presence of *Campylobacter* bacteria in test samples (such as clinical specimens and foods) by targeting cytolethal distending toxins of *Campylobacter* bacteria, including *Campylobacter coli*.

Background Art

Campylobacter bacteria are microorganisms that are pathogenic to humans as well as wild and domestic animals and that cause abortion and enteritis in animals and enteritis in humans. *Campylobacter jejuni* and *Campylobacter coli* are known to be causative bacteria of *Campylobacter* infection in humans. These bacteria are often referred to as food poisoning bacteria (see Non-patent Documents 1 and 2).

As of 2000, *Campylobacter* has been classified into 15 species and 9 subspecies. *C. jejuni* constitutes 95 to 99% of the bacteria that are isolated in human diarrhea cases, while other bacterial species, such as *C. coli*, constitutes only a few percent (see Non-patent Document 3). However, the carriage rate of *C. coli* is extremely high in pigs. In recent years, *Campylobacter* infection has been on an increasing trend with increasing meat imports mainly from Southeast Asia. In particular, the infection from chicken-related food, whose consumption has been growing as a result of problems with beef such as BSE and O-157, has rapidly increased.

In addition, while *Campylobacter fetus* has been known as an abortion-causing bacteria in sheep and bovine, it has only recently been reported to be involved in abortion and premature delivery in humans as well. *C. fetus* infection, resulting from eating raw liver or beef

contaminated with *C. fetus,* is associated with symptoms such as sepsis and meningitis. The primary source of *Campylobacter* infection in humans is chicken, which carries the bacteria at high densities in the intestinal tract (Non-patent Document 4).

Campylobacter bacteria are generally distributed at a high density in the digestive tract of animals, such as bovine, sheep, pig, and chicken, and thus recognized as causative bacteria of zoonosis. Most campylobacteriosis is thought to be caused by chicken. Infection may arise through direct contact with the above animals or their excrement, or through intake of or during processing of food, drinking water, milk, and such contaminated with the excrement. Furthermore, infection cases in facilities such as newborn nurseries have also been reported (see Non-patent Document 5).

Campylobacteriosis has a long incubation period, ranging 3 to 7 days. It is characterized by gastroenteritis symptoms, such as diarrhea (sometimes, bloody mucous diarrhea), abdominal pain, fever, nausea, vomiting, headache, chills, and feebleness. Although the lethality is low, newborn babies may develop systemic infection, such as sepsis and meningitis. In most cases, recovery takes several days to about one week. The general prognosis has a favorable course except in some immunodeficiency patients. However, it has been reported in recent years that patients may develop Guillain-Barre syndrome or Fischer syndrome, which are autoimmune diseases, after campylobacteriosis. The cases developed following campylobacteriosis generally tend to become severe, and the remission rate after one year of the onset is only about 60%.

Chemotherapy using antibiotics is performed for severe conditions or cases complicated by sepsis. The first choice drug is a macrolide, such as erythromycin. Due to natural resistance, cephem antibiotics are not expected to have therapeutic effects. Meanwhile, the increase in the number of bacteria resistant to new quinolone antibiotics has become a problem in recent years. Rapid identification of causative microorganisms after infection is important to conduct an appropriate treatment for campylobacteriosis and to prevent the expansion of infection by revealing the infection route. However, it is difficult to diagnose campylobacteriosis based on clinical symptoms alone, much less to identify *Campylobacter* and its species.

Campylobacter bacteria are microaerophiles. A culture of the bacteria requires a special medium such as Skirrow's medium, and a special apparatus (anaerobic jar or the like) to maintain the oxygen concentration at 3 to 10% for the absolute microaerophilic condition. In addition, the culture is time-consuming (2 to 3 days) as compared with other bacteria. Thus, it is difficult to achieve and maintain an isolation culture of *Campylobacter* bacteria. Furthermore, since *Campylobacter* bacteria easily die in the air, they must be tested within 2 to 3 hours after sample collection. Furthermore, since the incubation period of campylobacteriosis is long (3 to

7 days), the bacteria often cannot be isolated when bacterial identification in any foods concerned is carried out after the onset of the symptoms. Furthermore, *Campylobacter* bacteria have very strong infectivity, and have been reported to establish infection with only several hundreds of cells. Thus, it is extremely difficult to identify the source of infection.

One method of discriminative diagnosis between *C. jejuni* and *C. coli* involves testing hippurate hydrolysis. Specifically, the method is based on the fact that *C. jejuni* has the ability to hydrolyze hippurate while *C. coli* does not . However, this method is not exact because there some hippurate-negative *C. jejuni* species are known in the art (see Non-patent Document 6). Thus, the presence of *Campylobacter* bacteria can be confirmed only by estimating the presence of the bacteria from food intake history and symptoms, and by examining morphological and biological features of bacteria from colonies obtained by feces culture, which requires several days.

Thus, attempts have been made to identify *Campylobacter* bacteria and detect its toxin genes using, as rapid diagnostic methods that don't require cultivation, genetic diagnostic methods which utilize a DNA probe method or a PCR method using oligonucleotides. For example, the gene encoding rRNA has been generally used as a probe for *Campylobacter* bacteria (see Patent Document 1). The sequences of *Campylobacter* rRNA genes have already been published (see Non-patent Document 7). In addition, nucleic acid fragments for detecting *Campylobacter* bacteria are also known (see Patent Documents 2, 3, 4, 5, and 6). However, while these sequences may be used to detect *C. jejuni* and/or *C. coli*, they are not adequate to detect other *Campylobacter* bacteria. Furthermore, the current level of specificity is not sufficient.

A method for identifying *C. jejuni* by PCR, using oligonucleotides selected from the fla A gene of *C. coli* VC167, has also been reported (see Non-patent Document 8). Furthermore, the use of oligonucleotide primers to amplify a target sequence of superoxide dismutases of *C. jejuni* and *C. coli* has been reported in the literature (see Patent Document 7). However, these methods are incapable of discriminating between *C. jej*uni and *C. coli.*

Meanwhile, pathogenic factors of *Campylobacter* are being studied actively. Various factors, such as cell invasiveness, flagellin, and cholera toxin-like enterotoxin, have been reported as pathogenic factors of *Campylobacter* bacteria (see Non-patent Documents 9 and 10). Recently, cytolethal distending toxin (CDT) was discovered as a toxic factor from *C. jejuni* (Non-patent Document 11), and its relevance to the pathogenicity has attracted attention. For example, diarrheagenicity of the toxin has been reported in an animal model using recombinant *E. coli* that produces CDT of Shiga's bacillus (*Shigella dysenteriae*) (Non-patent Document 12).

CDT is a holotoxin composed of three subunits, called cdtA, cdtB, and cdtC, which are encoded by genes arranged in tandem. The active center of the toxin is in the cdtB subunit

having type I deoxyribonuclease-like activity, while the cdtA and cdtC subunits are thought to be involved in the adhesion to target cells. When the holotoxin acts on cells, the cells are distended, i.e. swollen, and finally killed. The toxin is thus named "cytolethal distending toxin".

The molecular mechanism is believed to be as follows. The cdtB subunit that constitutes the active center of the toxin translocates into a cell nucleus, and introduces nicks into chromosomal DNA by its type I deoxyribonuclease activity, thereby inducing DNA-damage response. The cell then arrests the cell cycle at G2/M phase to activate the gene repair system, and is then distended and killed (Non-patent Document 13). Furthermore, CDT has been found to act on a broad range of cells, including epithelial cells and immune cells. In particular, CDT is believed to act on human lymphocytes and induce apoptosis in them, which allows easy escape from immunity (Non-patent Document 14).

As described above, CDT has a unique molecular mechanism that is not found in the other toxins previously known. To date, the complete nucleotide sequence of CDT among *Campylobacter* bacteria has been determined for only *C. jejuni* (Non-patent Document 11).

Patent Document 1: Japanese Patent Application Kokai Publication No. (JP-A) S62-228096 (unexamined, published Japanese patent application)
Patent Document 2: JP-A H2-84200
Patent Document 3: JP-A H2-154700
Patent Document 4: JP-A H3-112498
Patent Document 5: JP-A H6-90795
Patent Document 6: JP-A H6-90796
Patent Document 7: JP-A 2000-316590
Non-patent Document 1: Blaser, et al, Ann. Intern. Med., 91:179 (1979)
Non-patent Document 2: Tauxe, R., American Society for Microbiology, Washington DC. pg. 9(1992)
Non-patent Document 3: Takahashi, M. et al, Infectious Diseases Weekly Report Japan, 3(6):10 (2001)
Non-patent Document 4: Simon, M. S. et al., 2003. Campylobacter infection. Diseases of Poultry, Iowa State Press, 615-630
Non-patent Document 5: Japanese Journal of Pediatric Medicine, 29:1219-1222 (1997)
Non-patent Document 6: Totten, et al, J. Clin. Microbiol., 25: 1747 (1987)
Non-patent Document 7: Romaniuk, P. J. et al, J. Bacteriol., 169: 2173 (1987)
Non-patent Document 8: Oyofo, et al, J. Clin. Microbiol., 30: 2613 (1992)
Non-patent Document 9: Mizuno, K. et al, Microbios., 78: 215 (1994)

Non-patent Document 10: Suzuki, S. et al, FEMS Immunol. Med. Microbiol., 8: 207 (1994)
Non-patent Document 11: Pickett, C. et al. Infect. Immun., 64: 2070 (1996)
Non-patent Document 12: Infect. Immun., 65: 428-433 (1997)
Non-patent Document 13: Science, 290: 354-357 (2000)
Non-patent Document 14: J. Biol. Chem., 276: 5296-5302 (2001)

Disclosure of the Invention

Problems to Be Solved by the Invention

As described in detail above, there is a need in the art for the rapid diagnosis of *Campylobacter* infection, despite the fact that the pathogenic factors of *Campylobacter* bacteria have not been fully elucidated. Conventionally, PCR primers for identifying bacterial species based on the serotype thereof, common primers for testing CDT production, and such have been used (J. Applied Microbiol., 94: 1003-1014 (2003)). However, such methods require the step of an enrichment culture, making the rapid detection of *Campylobacter* bacteria impossible.

Thus, an objective of the present invention is to provide CDT of *C. coli*, a *Campylobacter* species whose CDT nucleotide sequence has yet to be elucidated, and provide the polynucleotide encoding the CDT, in order to enable the rapid detection of *Campylobacter* bacteria through genetic diagnosis. Another objective of the present invention is to provide CDT of *C. fetus*, whose CDT nucleotide sequence has also yet to be elucidated, and provide the polynucleotide encoding the CDT.

Furthermore, another objective of the present invention is to provide methods that enable the rapid detection of *Campylobacter* bacteria, which target CDTs of *Campylobacter* bacteria, including *C. coli and C. fetus*, based on the findings obtained from the nucleotide sequences of *C. coli* and *C. fetus*.

Means to Solve the Problems

When cloning of the CDT genes is carried out using the restriction enzyme *Hin*dIII, its full length cannot be isolated because its coding region contains *Hin*dIII sites. Meanwhile, common restriction enzymes, such as *Eco*RI, *Pst*I, *Kpn*I, *Xba*I, *Bam*HI, *Sal*I, and *Xho*I, do not yield fragments with an adequate length (3 to 5 kb) for cloning of the cdt genes. As a result of various studies, the present inventors finally succeeded in cloning the complete cdt genes without any cleavage in their internal sequences by selecting a partial digestion condition wherein the cdt gene is not completely digested with *Hin*dIII.

The present inventors also compared the *C. coli* CDT with CDTs of *C. jejuni* and *C. fetus* and developed primers common to the three *Campylobacter* bacteria and primers specific to each of the bacteria. The inventors then demonstrated that these primers were applicable to

multiplex PCR that simultaneously allows for rapid and convenient determination of the presence of *Campylobacter* CDT and identification of species, and that they can also be used in PCR-RFLP-based typing.

Specifically, the present invention encompasses the following technical embodiments:

(1) a polynucleotide encoding a cytolethal distending toxin, which is any one of:

(a) a polynucleotide encoding a polypeptide comprising the amino acid sequence of any one of SEQ ID NOs: 2 to 4;

(b) a polynucleotide comprising the coding region in the nucleotide sequence of SEQ ID NO: 1;

(c) a polynucleotide encoding a polypeptide comprising an amino acid sequence with a substitution, deletion, addition, and/or insertion of one or more amino acids in any one of the amino acid sequences of SEQ ID NOs: 2 to 4; and

(d) a polynucleotide that hybridizes to DNA comprising the nucleotide sequence of SEQ ID NO: 1 under a stringent condition;

(2) a vector comprising the polynucleotide of (1);

(3) a host cell carrying the polynucleotide of (1) or the vector of (2);

(4) a polypeptide encoded by the polynucleotide of (1);

(5) a method for producing the polypeptide of (4), which comprises the step of culturing the host cell of (3) and collecting the produced polypeptide from the host cell or the culture supernatant;

(6) an antibody that binds to the polypeptide of (4);

(7) a method for detecting the presence of *Campylobacter coli*, *Campylobacter jejuni*, and *Campylobacter fetus* in a test sample, wherein the method comprises the steps of:

(a) conducting a polymerase chain reaction on the test sample using a mixture of primer pairs specific to each of genomic DNAs encoding the cytolethal distending toxins of these bacteria; and

(b) determining the presence of these bacteria based on the presence or molecular weight of amplified fragments from the genomic DNAs encoding the cytolethal distending toxins of the bacteria;

(8) a method for detecting the presence of *Campylobacter coli*, *Campylobacter jejuni*, and *Campylobacter fetus* in a test sample, wherein the method comprises the steps of:

(a) conducting a polymerase chain reaction on the test sample using a common primer pair which can amplify genomic DNAs encoding the cytolethal distending toxins of these bacteria;

(b) conducting a polymerase chain reaction using the genomic DNA amplified in step (a) as a template and a mixture of primer pairs specific to each of the genomic DNAs encoding cytolethal distending toxins of the bacteria; and

(c) determining the presence of the bacteria based on the presence or molecular weight of amplified fragments from the genomic DNAs encoding the cytolethal distending toxins of the bacteria;

(9) the method of (8), wherein the common primer pair is a primer pair selected from SEQ ID NOs: 7 to 10 and 47 to 50, or a primer pair which can amplify the same genomic DNA region as amplified with said primer pair;

(10) the method of (7) or (8), wherein the method uses (a) to (c) as the mixture of specific primer pairs:

(a) a primer pair selected from SEQ ID NOs: 13, 14, and 28 to 36 to amplify the genomic DNA encoding the cytolethal distending toxin of *Campylobacter coli*, or a primer pair which can amplify the same genomic DNA region as amplified with said primer pair;

(b) a primer pair selected from SEQ ID NOs: 11, 12, and 17 to 27 to amplify the genomic DNA encoding the cytolethal distending toxin of *Campylobacter jejuni*, or a primer pair which can amplify the same genomic DNA region as amplified with said primer pair; and

(c) a primer pair selected from SEQ ID NOs: 15, 16, and 37 to 46 to amplify the genomic DNA encoding the cytolethal distending toxin of *Campylobacter fetus*, or a primer pair which can amplify the same genomic DNA region as amplified with said primer pair;

(11) a method for detecting the presence of *Campylobacter coli*, *Campylobacter jejuni*, and *Campylobacter fetus* in a test sample, wherein the method comprises the steps of:

(a) conducting a polymerase chain reaction on the test sample using a common primer pair which can amplify genomic DNAs encoding the cytolethal distending toxins of these bacteria;

(b) digesting the genomic DNAs amplified in step (a) with a restriction enzyme; and

(c) determining the presence of the bacteria based on the molecular weight of a DNA fragment resulting from the digestion;

(12) the method of (11), wherein the restriction enzyme is selected from the group consisting of: *Sau*3AI, *Dsa* I, *Mbo* I, *Rsa* I, *Eco*RI, *Hin*f I, *Nde* I, *Pst* I, *Xba* I, and *Xho* II;

(13) the method of (11), wherein the common primer pair is a primer pair selected from SEQ ID NOs: 7 to 10 and 47 to 50, or a primer pair which can amplify the same genomic DNA region as amplified with said primer pair;

(14) a kit used in the method of (7), which comprises an instruction manual and a mixture of primer pairs specific to each of genomic DNAs encoding the cytolethal distending toxins of *Campylobacter coli*, *Campylobacter jejuni*, and *Campylobacter fetus*;

(15) the kit of (14) wherein the mixture of specific primer pairs is as follows:

(a) a primer pair selected from SEQ ID NOs: 13, 14, and 28 to 36 to amplify the genomic DNA encoding the cytolethal distending toxin of *Campylobacter coli*, or a primer pair

which can amplify the same genomic DNA region as amplified with said primer pair;

(b) a primer pair selected from SEQ ID NOs: 11, 12, and 17 to 27 to amplify the genomic DNA encoding the cytolethal distending toxin of *Campylobacter jejuni*, or a primer pair which can amplify the same genomic DNA region as amplified with said primer pair; and

(c) a primer pair selected from SEQ ID NOs: 15, 16, and 37 to 46 to amplify the genomic DNA encoding the cytolethal distending toxin of *Campylobacter fetus*, or a primer pair which can amplify the same genomic DNA region as amplified with said primer pair;

(16) a kit used in the method of claim 8, which comprises an instruction manual and:

(a) a mixture of primer pairs specific to each of genomic DNAs encoding cytolethal distending toxins of *Campylobacter coli*, *Campylobacter jejuni*, and *Campylobacter fetus*; or

(b) a common primer pair which can amplify the genomic DNAs encoding the cytolethal distending toxins of *Campylobacter coli*, *Campylobacter jejuni*, and *Campylobacter fetus*;

(17) the kit of (16), wherein the mixture of specific primer pairs is as follows:

(a) a primer pair selected from SEQ ID NOs: 13, 14, and 28 to 36 to amplify the genomic DNA encoding the cytolethal distending toxin of *Campylobacter coli*, or a primer pair which can amplify the same genomic DNA region as amplified with said primer pair;

(b) a primer pair selected from SEQ ID NOs: 11, 12, and 17 to 27 to amplify the genomic DNA encoding the cytolethal distending toxin of *Campylobacter jejuni*, or a primer pair which can amplify the same genomic DNA region as amplified with the primer pair; and

(c) a primer pair selected from SEQ ID NOs: 15, 16, and 37 to 46 to amplify the genomic DNA encoding the cytolethal distending toxin of *Campylobacter fetus*, or a primer pair which can amplify the same genomic DNA region as amplified with the primer pair;

(18) the kit of (16), wherein the common primer pair is a primer pair selected from SEQ ID NOs: 7 to 10 and 47 to 50, or a primer pair which can amplify the same genomic DNA region as amplified with said primer pair;

(19) a kit used in the method of (11), which comprises an instruction manual and a common primer pair which can amplify genomic DNAs encoding the cytolethal distending toxins of *Campylobacter coli*, *Campylobacter jejuni*, and *Campylobacter fetus*; and

(20) the kit of (19), wherein the common primer pair is a primer pair selected from SEQ ID NOs: 7 to 10 and 47 to 50, or a primer pair which can amplify the same genomic DNA region as amplified with said primer pair.

Herein, the phrase "cytolethal distending toxins" (CDTs or CLDTs) refers to toxic factors belonging to the group of proteinaceous type A-B holotoxins. The cytolethal distending toxin has a subunit structure consisting of three subunits A, B, and C. It is believed that subunit B is the active site unit of the toxin and subunits A and B are involved in cell adhesion. When

the toxin acts on cells, it causes cell deformation such as cell swelling, and finally leads to cell death. Cell deformation such as cell swelling is also observed when heat-labile enterotoxin (LT), which is produced by toxigenic *E. coli*, or the like is experimentally allowed to act on cells. When the toxin is removed, however, the cells recover and survive. In contrast, cells do not recover but instead are killed, even when CDT is removed.

The term "polynucleotide" as used herein refers to a ribonucleotide or deoxyribonucleotide, or a polymer made up of a number of bases or base pairs. Polynucleotides include single-stranded DNAs as well as double-stranded DNAs. Polynucleotides herein may include both unmodified, naturally-occurring polynucleotides and modified polynucleotides. Tritylated bases and special bases, such as inosine, are examples of modified bases.

The term "polypeptide" as used herein refers to a polymer made up of a number of amino acids. Therefore, oligopeptides and proteins are also included within the concept of polypeptides. Polypeptides include both unmodified, naturally-occurring polypeptides and modified polypeptides. Examples of polypeptide modifications include acetylation; acylation; ADP-ribosylation; amidation; covalent binding with flavin; covalent binding with heme moieties; covalent binding with nucleotides or nucleotide derivatives; covalent binding with lipids or lipid derivatives; covalent binding with phosphatidylinositols; cross-linkage; cyclization; disulfide bond formation; demethylation; covalent cross linkage formation; cystine formation pyroglutamate formation; formylation; g-carboxylation; glycosylation; GPI-anchor formation; hydroxylation; iodination; methylation; myristoylation; oxidation; proteolytic treatment; phosphorylation; prenylation; racemization; selenoylation; sulfation; transfer RNA-mediated amino acid addition to a protein such as arginylation; ubiquitination; and the like.

The term "isolate" as used herein refers to a substance (for example, a polynucleotide or polypeptide) removed from its original environment (for example, the natural environment for a naturally-occurring substance) and "artificially" changed from its natural state. "Isolated" compounds refer to compounds including those present in samples that are substantially abundant with a subject compound, and/or those present in samples wherein the subject compound is partly or substantially purified. Herein, the term "substantially purified" refers to compounds (for example, polynucleotides or polypeptides) that are isolated from the natural environment and in which at least 60%, preferably 75%, and most preferably 90% of the other components associated with the compound in nature are absent.

The term "mutation" as used herein refers to changes to the amino acids of an amino acid sequence, or changes to the bases in a nucleotide sequence (that is, substitution, deletion, addition, or insertion of one or more amino acids or nucleotides). Therefore, the term "mutant" as used herein refers to amino acid sequences wherein one or more amino acids are changed, or

nucleotide sequences wherein one or more nucleotides are changed. Nucleotide sequence changes in the mutant may change the amino acid sequence of the polypeptide encoded by the standard polynucleotide, or not. The mutant may be one that exists in nature, such as an allelic mutant, or one not yet identified in nature. The mutant may be conservatively altered, wherein substituted amino acids retain structural or chemical characteristics similar to those of the original amino acid. Rarely, mutants may be substituted non-conservatively. Computer programs known in the art, such as DNA STAR software, can be used to decide which or how many amino acid residues to substitute, insert, or delete without inhibiting biological or immunological activities.

"Deletion" is a change to either an amino acid sequence or nucleotide sequence, wherein one or more amino acid residues or nucleotide residues are missing as compared with the amino acid sequence of a naturally occurring cytolethal distending toxin polypeptide, or a nucleotide sequence encoding the same.

"Insertion" or "addition" is a change to either an amino acid sequence or nucleotide sequence, wherein one or more amino acid residues or nucleotide residues are added as compared with the amino acid sequence of a naturally-occurring cytolethal distending toxin polypeptide, or a nucleotide sequence encoding the same.

"Substitution" is a change to either an amino acid sequence or nucleotide sequence, wherein one or more amino acid residues or nucleotide residues are changed to different amino acid residues or nucleotide residues, as compared to the amino acid sequence of a naturally-occurring cytolethal distending toxin polypeptide, or a nucleotide sequence encoding the same.

The term "hybridize" as used herein refers to a process wherein a nucleic acid chain binds to its complementary chain through the formation of base pairs.

Brief Description of the Drawings

Fig. 1 is a photograph showing a result of PCR with GNW and LPF-D primers using *C. coli* Co 1-192 cell extract as a template. Arrow 1 indicates the bands resulting from amplification of the cdt region (about 1.5 Kb); the bands (800 bp) of arrow 2 are secondary bands derived from cdtB, which were amplified since the GNW primers were mixed primers.

Fig. 2 is a photograph showing a result of hybridization after digestion of genomes from *C. coli* Co 1-192 cells with the restriction enzyme *Hin*dIII.

Fig. 3 is a photograph showing a result of PCR using common primer pair 1. CDT-derived bands are seen at about 1.9 kbp in lanes 2 to 6.

Fig. 4 is a photograph showing a result of PCR for various *C. jejuni* strains using common primer pair 2. CDT-derived bands are seen at about 720 bp.

Fig. 5 is a photograph showing a result of PCR for various *C. jejuni* and *C. coli* strains using common primer pair 2.

Fig. 6 is a photograph showing a result of PCR for *C. jejuni*, *C. coli* and *C. fetus* strains using common primer pair 2.

Fig. 7 is a photograph showing a result of multiplex PCR for *C. jejuni*, *C. coli*, and *C. fetus* strains using specific primers. CDT-specific amplified fragments unique to each species were detected (*C. jejuni*, 750 bp; *C. coli*, 400 bp; *C. fetus*, 530 bp).

Fig. 8 is a photograph showing a result of PCR-RFLP for *C. jejuni*, *C. coli*, and *C. fetus* strains using common primer pair 1.

Fig. 9 is a set of photographs showing a result of multiplex PCR for various strains of *C. jejuni*, *C. coli*, and *C. fetus* using specific primers. CDT-specific amplified fragments unique to each species were detected (*C. jejuni*, 750 bp; *C. coli*, 400 bp; *C. fetus*, 530 bp).

Fig. 10 is a photograph showing a result of hybridization after digestion of genomes from *C. fetus* Col-187 cells with the restriction enzyme *Hin*dIII.

Fig. 11 is a photograph showing a result of PCR using common primers for cdtA and cdtC. Bands derived from cdtA are seen at about 550 bp in lanes 2 to 8; and bands derived from cdtC are seen at about 320 bp in lanes 10 to 16.

Fig. 12 is a set of photographs showing a result of PCR for various strains of *Campylobacter* species using common primers for cdtA. Bands derived from cdtA are seen at about 550 bp.

Fig. 13 is a set of photographs showing a result of PCR for various strains of *Campylobacter* species using common primers for cdtC. Bands derived from cdtC are seen at about 320 bp.

Fig. 14 is a photograph showing a result of multiplex PCR for *C. jejuni*, *C. coli*, and *C. fetus* strains using primers specific to cdtA and cdtC. Amplified fragments specific to cdtA (*C. jejuni*, 630 bp; *C. coli*, 330 bp; *C. fetus*, 490 bp) and to cdtC (*C. jejuni*, 500 bp; *C. coli*, 400 bp; *C. fetus*, 300 bp) unique to each species are detected.

Fig. 15 is a set of photographs showing a result of multiplex PCR for *C. jejuni*, *C. coli*, and *C. fetus* strains using cdtA-specific primers. CdtA-specific amplified fragments unique to each species were detected.

Fig. 16 is a set of photographs showing a result of multiplex PCR for *C. jejuni*, *C. coli*, and *C. fetus* strains using cdtC-specific primers. CdtC-specific amplified fragments unique to each species were detected.

Fig. 17 shows the ORF of *C. jejuni* CDT and the primer annealing regions.

Fig. 18 shows the ORF of *C. coli* CDT and the primer annealing regions.

Fig. 19 shows the ORF of *C. fetus* CDT and the primer annealing regions.

Best Mode for Carrying Out the Invention

<Polynucleotide>

The present invention provides a polynucleotide encoding the cytolethal distending toxin of *Campylobacter coli*. The polynucleotide sequence encoding the cytolethal distending toxin of *C. coli*, which was identified by the present inventors and is encompassed by the present invention, is set forth in SEQ ID NO: 1. The amino acid sequences of the three polypeptides encoded by the polynucleotide are set forth in SEQ ID NOs: 2 to 4. SEQ ID NOs: 2, 3, and 4 correspond to the amino acid sequences of cdtA, cdtB, and cdtC, respectively.

The present invention also provides a polynucleotide encoding the cytolethal distending toxin of *Campylobacter fetus*. The polynucleotide sequence encoding the cytolethal distending toxin of *C. fetus*, which was identified by the present inventors and is encompassed by the present invention, is set forth in SEQ ID NO: 51. The amino acid sequences of the three polypeptides encoded by the polynucleotide are set forth in SEQ ID NOs: 52 to 54; SEQ ID NOs: 52, 53, and 54 correspond to the amino acid sequences of cdtA, cdtB, and cdtC, respectively.

The polynucleotides of the present invention include polynucleotides encoding the polypeptides having the amino acid sequences of SEQ ID NOs: 2 to 4; polynucleotides including the coding region in the nucleotide sequence of SEQ ID NO: 1, *i.e.* any one of the nucleotide sequences of position 1 to 777, 802 to 1605, and 1615 to 2187 in SEQ ID NO: 1; and polynucleotides that have a nucleotide sequence different from the nucleotide sequence of SEQ ID NO: 1 but yet encode the polypeptides having the amino acid sequences of SEQ ID NOs: 2 to 4 due to the degeneracy of the genetic code.

The polynucleotides of the present invention also include polynucleotides encoding the polypeptides having the amino acid sequences of SEQ ID NOs: 52 to 54; polynucleotides including the coding region in the nucleotide sequence of SEQ ID NO: 51, *i.e.* any one of the nucleotide sequences of position 1 to 702, 778 to 1629, and 1615 to 2187 in SEQ ID NO: 51; and polynucleotides that have a nucleotide sequence different from the nucleotide sequence of SEQ ID NO: 51 but yet encode the polypeptides having the amino acid sequences of SEQ ID NOs: 52 to 54 due to the degeneracy of the genetic code.

The polynucleotides of the present invention further include polynucleotides that encode polypeptides functionally equivalent to polypeptides encoded by the above polynucleotides and have a nucleotide sequence with an identity of at least 40% or higher, preferably 60% or higher, more preferably 80% or higher, even more preferably 90% or higher, still more preferably 95% or higher, yet more preferably 97% or higher (for example, 98 to 99%) to the entire sequence of the polynucleotide. The nucleotide sequence identity can be determined, for example, using the

algorithm BLAST by Karlin and Altschul (Proc. Natl. Acad. Sci. USA 87:2264-2268, 1990; Proc. Natl. Acad. Sci. USA 90:5873-5877, 1993). A program called BLASTN has been developed based on this algorithm (Altschul et al. J. Mol. Biol.215:403-410, 1990). When nucleotide sequences are analyzed by BLASTN, the parameters are set, for example, as follows: score = 100; wordlength = 12. When BLAST and Gapped BLAST programs are used, default parameters are used for each program. The specific techniques for these analytical methods are known (http://www.ncbi.nlm.nih.gov.). The polynucleotides of the present invention include polynucleotides having nucleotide sequences complementary to the above polynucleotide sequences.

The polynucleotides of the present invention can be obtained through standard cloning and screening methods from natural sources, such as genomic DNA in bacterial cells. Alternatively, the polynucleotides can be obtained from cDNA libraries derived from mRNA in bacterial cells. The polynucleotides can also be synthesized using known techniques that are commercially available.

Polynucleotides having nucleotide sequences with significant homology to the polynucleotide sequences identified by the present inventors (e.g., SEQ ID NOs: 1 and 51) can be prepared, for example, using hybridization techniques (Current Protocols in Molecular Biology edit. Ausubel et al. (1987) Publish. John Wiley & Sons Section 6.3-6.4) and gene amplification techniques (PCR) (Current protocols in Molecular Biology edit. Ausubel et al. (1987) Publish. John Wiley & Sons Section 6.1-6.4). Specifically, based on the polynucleotide sequences identified by the present inventors (e.g., SEQ ID NOs: 1 and 51) or portions thereof, DNA highly homologous to the sequences can be isolated using known hybridization techniques. Alternatively, polynucleotides highly homologous to the polynucleotide sequences can be isolated by gene amplification techniques, using primers designed based on portions of the polynucleotide sequences identified by the present inventors (e.g., SEQ ID NOs: 1 and 51). Thus, the present invention includes polynucleotides that hybridize to the polynucleotide having the nucleotide sequence of SEQ ID NO: 1 under a stringent condition. A stringent hybridization condition is typically the condition of 1x SSC, 0.1% SDS, and 37°C. A more stringent condition is the condition of 0.5x SSC, 0.1% SDS, and 42°C. A still more stringent condition is the condition of 0.2xSSC, 0.1% SDS, and 65°C. As the hybridization condition is more stringent as described above, DNA having higher homology to the probe sequence is expected to be isolated. However, the above combinations of SSC, SDS, and temperature condition are only exemplary. Those skilled in the art can achieve the same stringency as described above by appropriately combining the above or other factors (for example, probe concentration and length, and reaction time for hybridization) which determines the degree of hybridization stringency.

Polynucleotides including the nucleotide sequences with significant homology to the polynucleotide sequences identified by the present inventors can also be prepared by methods for introducing mutations into the nucleotide sequences of SEQ ID NOs: 1 and 51 (for example, site directed mutagenesis (Current Protocols in Molecular Biology edit. Ausubel et al. (1987) Publish. John Wiley & Sons Section 8.1-8.5)). Such polynucleotides may also be generated by naturally-occurring mutations. The present invention includes polynucleotides encoding the polypeptides having an amino acid sequence wherein one or more amino acids is substituted, deleted, inserted and/or added in the amino acid sequences of SEQ ID NOs: 2 to 4 or 52 to 54 due to such nucleotide sequence mutations.

When the polynucleotides of the present invention are used to produce the polypeptides of the present invention, the polynucleotides include coding sequences for the mature polypeptides or fragments thereof alone, or coding sequences for the mature polypeptides or fragments thereof which are located in the same reading frame as other coding sequences (for example, leader or secretory sequence, pre-, pro-, or prepro-protein sequence, or sequences encoding other fusion peptide portions). For example, marker sequences that facilitate purification of fusion polypeptides may be encoded. In this embodiment of the present invention, preferred examples of marker sequences include, for example, hexa-histidine peptide or Myc tag which is provided by pcDNA3.1/Myc-His vector (Invitrogen) and described in Gentz et al., Proc. Natl. Acad. Sci. USA (1989) 86:821-824. The polynucleotide may also include 5' and 3' non-coding sequences, for example, transcribed but untranslated sequences, splicing and polyadenylation signals, ribosome-binding site, and mRNA-stabilizing sequence.

<Polypeptide>

The present invention provides the polypeptide of the cytolethal distending toxin of *Campylobacter coli* identified by the present inventors. The present invention also provides polypeptides functionally equivalent to the polypeptide identified by the present inventors. Herein, "functionally equivalent" means that a polypeptide of interest has characteristics of cytolethal distending toxin equivalent to that of the polypeptide identified by the present inventors.

The present invention also provides the polypeptide of the cytolethal distending toxin of *Campylobacter fetus* identified by the present inventors. The present invention further provides polypeptides functionally equivalent to the polypeptide identified by the present inventors. Herein, "functionally equivalent" means that a polypeptide of interest has characteristics of cytolethal distending toxin equivalent to that of the polypeptide identified by the present inventors.

Introducing mutations into the amino acid sequence of proteins is one means for

preparing polypeptides functionally equivalent to the polypeptides identified by the inventors. Such methods include, for example, site-directed mutagenesis (Current Protocols in Molecular Biology, edit. Ausubel et al. (1987) Publish. John Wiley & Sons Section 8.1-8.5). Amino acid mutation in polypeptides may also occur in nature. The present invention includes mutant proteins, regardless of whether artificially or naturally produced, that include the amino acid sequence identified by the inventors (e.g., SEQ ID NO: 2 to 4 and 52 to 54), wherein one or more amino acid residues are altered by substitution, deletion, insertion, and/or addition, yet which are functionally equivalent to the polypeptides identified by the present inventors.

From the viewpoint of conserving the protein's functions, an amino acid residue used for substitution preferably has properties similar to the substituted amino acid residue (conservative substitution). For example, Ala, Val, Leu, Ile, Pro, Met, Phe, and Trp are all classified as non-polar amino acids, and are considered to have similar properties. Further, examples of uncharged amino acids are Gly, Ser, Thr, Cys, Tyr, Asn, and Gln. Moreover, examples of acidic amino acids are Asp and Glu, and those of basic amino acids are Lys, Arg, and His.

There are no limitations as to the number and site of the amino acid mutations of these polypeptides, so long as the mutated polypeptides retain a function of the original polypeptide. The number of mutations may be typically less than 10%, preferably less than 5%, and more preferably less than 1% of the total amino acid residues.

Other means for preparing polypeptides functionally equivalent to the polypeptides identified by the inventors include methods that utilize hybridization techniques or gene amplification techniques. More specifically, those skilled in the art can obtain polypeptides functionally equivalent to the polypeptides determined by the present inventors by isolating highly homologous DNAs from DNA samples derived from organisms of the same or different species using hybridization techniques (Current Protocols in Molecular Biology, edit. Ausubel et al. (1987) Publish. John Wiley & Sons Section 6.3-6.4) based on the DNA sequence encoding the polypeptides identified by the inventors (SEQ ID NO: 1 and 51). Thus, such polypeptides, encoded by DNAs hybridizing to the DNAs encoding the polypeptides identified by the inventors, which are functionally equivalent to the polypeptides identified by the inventors, are also included in the polypeptides of this invention.

Hybridization stringencies required to isolate a DNA encoding a polypeptide functionally equivalent to the polypeptides identified by the inventors are normally "1x SSC, 0.1% SDS, 37°C" or such, with more stringent conditions being "0.5x SSC, 0.1% SDS, 42°C" or such, and even more stringent conditions being "0.2x SSC, 0.1% SDS, 65°C" or such. DNAs with higher homology to the probe sequence are expected to be isolated at higher stringencies. However, the above-mentioned combinations of SSC, SDS, and temperature conditions are only

examples, and those skilled in the art can achieve the same stringencies as described above by appropriately combining the above-mentioned factors or other parameters which determine hybridization stringency (for example, probe concentration, probe length, reaction time of hybridization, etc.).

The polypeptides encoded by DNAs isolated using such hybridization techniques normally have amino acid sequences highly homologous to the polypeptides identified by the present inventors. Herein, high homology indicates sequence identity of at least 40% or more, preferably 60% or more, more preferably 80% or more, still more preferably 90% or more, further still more preferably at least 95% or more, and yet more preferably at least 97% or more (for example, 98% to 99%). Homology of amino acid sequences can be determined, for example, using the algorithm BLAST of Karlin and Altschul (Proc. Natl. Acad. Sci. USA 87: 2264-2268 (1990); Proc. Natl. Acad. Sci. USA 90: 5873-5877 (1993)). Based on this algorithm, a program referred to as BLASTX has been developed (Altschul et al., J. Mol. Biol. 215: 403-410 (1990)). When amino acid sequences are analyzed using BLASTX, parameters are set, for example, at score = 50 and wordlength = 3, while when using BLAST and Gapped BLAST programs, default parameters of each program are used. Specific techniques for these analytical methods are well known in the field.

Gene amplification techniques (PCR) (Current Protocols in Molecular Biology, edit. Ausubel et al. (1987) Publish. John Wiley & Sons Section 6.1-6.4) can be utilized to obtain polypeptides functionally equivalent to the polypeptides isolated by the present inventors, based on DNA fragments isolated as DNAs highly homologous to the DNA sequences encoding the polypeptides isolated by the present inventors. This can be achieved by designing primers based on a part of the DNA sequence encoding the polypeptides identified by the inventors (SEQ ID NO: 1 and 51).

<Polypeptide fragments>

The present invention also provides fragments of the polypeptides of this invention. These fragments are polypeptides having amino acid sequences that are partly, but not entirely, identical to the above polypeptides of this invention. The polypeptide fragments of this invention usually include eight amino acid residues or more, and preferably twelve amino acid residues or more (for example, 15 amino acid residues or more). Examples of preferred fragments include truncation polypeptides, such as amino acid sequences that lack a series of amino acid residues including either the amino terminus or carboxyl terminus, or two series of amino acid residues, one including the amino terminus and the other including the carboxyl terminus. Furthermore, fragments featuring structural or functional characteristics are also preferable, and include those having α-helix and α-helix forming regions, β-sheet and β-sheet

forming regions, turn and turn-forming regions, coil and coil-forming regions, hydrophilic regions, hydrophobic regions, α-amphipathic regions, β-amphipathic regions, variable regions, surface forming regions, substrate-binding regions, and high antigenicity index regions. Biologically active fragments are also preferred. Biologically active fragments mediate the activities of the polypeptides of this invention, and include those that have a similar or improved activity, or a reduced undesirable activity. For example, fragments that are antigenic or immunogenic in animals, especially humans, are included. These polypeptide fragments preferably retain a biological activity, such as antigenicity, of the polypeptides of this invention. Mutants of specific sequences or fragments also constitute an aspect of this invention. Preferred mutants are those that differ from the subject polypeptide due to replacement with conservative amino acids, namely, those in which a residue is substituted with another residue of similar properties. Typical substitutions are those between Ala, Val, Leu, and Ile; Ser and Thr; acidic residues Asp and Glu, Asn, and Gln; basic residues Lys and Arg; or aromatic residues Phe and Tyr.

<Production of polypeptides>

Polypeptides of this invention may be produced by any appropriate method. Such polypeptides include isolated naturally-occurring polypeptides, and polypeptides which are produced by gene recombination, synthesis, or by a combination thereof. Procedures for producing these polypeptides are well known in the art. Recombinant polypeptides may be prepared, for example, by transferring a vector, inserted with a polynucleotide of the present invention, into an appropriate host cell, and purifying the polypeptide expressed within the resulting transformant. On the other hand, naturally occurring polypeptides can be prepared, for example, using affinity columns wherein antibodies against a polypeptide of this invention (described below) are immobilized (Current Protocols in Molecular Biology, edit. Ausubel et al. (1987) Publish. John Wiley & Sons, Section 16.1-16.19). Antibodies for affinity purification may be either polyclonal or monoclonal antibodies. The polypeptides of this invention may be also prepared by *in vitro* translation methods (for example, see "On the fidelity of mRNA translation in the nuclease-treated rabbit reticulocyte lysate system." Dasso, M. C. and Jackson, R. J. (1989) NAR 17: 3129-3144), and such. The polypeptide fragments of this invention can be produced, for example, by cleaving the polypeptides of the present invention with appropriate peptidases.

<Probes, primers>

The present invention provides nucleotides with a chain length of at least 15 nucleotides, which are complementary to a polynucleotide identified by the present inventors (e.g., a

polynucleotide having the nucleotide sequence of SEQ ID NO: 1 or a complementary strand thereof, and a polynucleotide having the nucleotide sequence of SEQ ID NO:51 or a complementary strand thereof). Herein, the term "complementary strand" is defined as the other strand of a double-stranded nucleic acid composed of A:T (A:U in case of RNA) and G:C base pairs. In addition, the term "complementary" encompasses not only complete matching within a continuous region of at least 15 sequential nucleotides, but also homology of at least 70%, preferably at least 80%, more preferably 90%, and most preferably 95% or higher within that region. Homology may be determined using an algorithm described herein. Probes and primers for detection or amplification of the polynucleotides of the present invention are included in these polynucleotides. Typical polynucleotides used as primers are 15 to 100 nucleotides long, and preferably 15 to 35 nucleotides long. Alternatively, polynucleotides used as probes are nucleotides at least 15 nucleotides in length, and preferably at least 30 nucleotides. They include at least a portion or an entire sequence of a DNA of the present invention. When using the nucleotides of the present invention as primers, the nucleic acid amplification reaction is not particularly limited, so long as a desired amplification product can be obtained. For example, the reaction may be selected from DNA amplification reactions such as polymerase chain reaction (PCR), ICAN, LAMP, SDA, and LCR, and RNA amplification reactions such as NASBA. A preferred method is PCR.

In one embodiment, such nucleotides are those specific to a DNA encoding a polypeptide of the present invention. The term "specific" refers to hybridizing under normal hybridization conditions, preferably stringent conditions, with DNA encoding a certain polypeptide, but not with DNAs encoding other polypeptides. Preferred embodiments are polynucleotides that hybridize to the genomic DNA encoding the cytolethal distending toxin of *Campylobacter coli* (SEQ ID NO: 1) but not to genomic DNAs encoding the cytolethal distending toxins of *Campylobacter jejuni* and *Campylobacter fetus*. Such polynucleotides include, for example, primer pairs selected from SEQ ID NOs: 13, 14, 28 to 36, 70, 71, 76, and 77. Alternatively, preferred embodiments are polynucleotides that hybridize to the genomic DNA encoding the cytolethal distending toxin of *Campylobacter fetus* (SEQ ID NO: 51) but not to genomic DNAs encoding the cytolethal distending toxins of *Campylobacter jejuni* and *Campylobacter coli*. Such polynucleotides include, for example, primer pairs selected from SEQ ID NOs: 15, 16, 37 to 46, 72, 73, 78, and 79.

In addition, with the identification of the genomic DNA encoding the cytolethal distending toxin of *Campylobacter coli* (SEQ ID NO: 1) in the Examples, the present inventors found nucleotide sequences specific to genomic DNAs encoding the cytolethal distending toxins of *Campylobacter jejuni* and *Campylobacter fetus*. Thus, the present invention also provides primer pairs specific to the genomic DNA encoding the cytolethal distending toxin of

Campylobacter jejuni and those specific to the genomic DNA encoding the cytolethal distending toxin of *Campylobacter fetus*. The primers specific to the genomic DNA encoding the cytolethal distending toxin of *Campylobacter jejuni* include, but not limited to, for example, the primers of SEQ ID NOs: 11, 12, and 17 to 27. The primers specific to the genomic DNA encoding the cytolethal distending toxin of *Campylobacter fetus* include, but not limited to, for example, the primers of SEQ ID NOs: 15, 16, and 37 to 46.

In addition, with the identification of the genomic DNAs encoding the cytolethal distending toxins of *Campylobacter coli* (SEQ ID NO: 1) and *Campylobacter fetus* (SEQ ID NO: 51) in the Examples, the present inventors found common primers for the genomic DNAs encoding the cytolethal distending toxins of *Campylobacter coli*, *Campylobacter jejuni*, and *Campylobacter fetus* (primers that can amplify all genomic DNAs encoding the cytolethal distending toxins of these bacteria). The present invention also provides such common primers. Preferred common primers include, for example, primers of SEQ ID NOs: 64 and 65 (to amplify ctdA DNA), primers of SEQ ID NOs: 7 to 10 and 47 to 50 (to amplify ctdB DNA), and primers of SEQ ID NOs: 66 and 67 (to amplify ctdC DNA).

Those skilled in the art can appropriately prepare primers that include one or more nucleotides different from the above primers but can amplify the same genomic DNA regions as amplified with the above primers. Genomic DNA regions to which the above primers anneal are shown in Figs. 17 to 19. The present invention also provides such mutant primers. As described above, nucleic acid amplification reactions to which the primers of the present invention are applicable are not particularly limited, so long as it yields desired amplification products. For example, the reaction can be selected from DNA amplification reactions such as PCR (polymerase chain reaction), ICAN, LAMP, SDA, and LCR, and RNA amplification reactions such as NASBA. A preferred method is PCR. Based on the above primers, those skilled in the art can design mutant primers adequate for nucleic acid amplification methods to be performed. Such mutant primers can be synthetically prepared. It can be readily assessed whether mutant primers can amplify the same genomic DNA region as amplified with the original primers, by conducting a nucleic acid amplification reaction using the mutant primers and analyzing the amplification products.

These primers can be preferably used to detect *Campylobacter* bacteria in test samples.

<Production of vectors, host cells, and polypeptides>

The present invention also provides methods for producing vectors carrying polynucleotides of the present invention, host cells retaining the polynucleotides or said vectors of the present invention, and polypeptides of the present invention utilizing said host cells.

The vectors of the present invention are not limited, so long as the DNAs inserted in the

vectors are stably retained. For example, pBluescript vector (Stratagene) is a preferable cloning vector when using *E. coli* as a host. When using vectors to produce the polypeptides of the present invention, expression vectors are particularly useful. These expression vectors are not specifically limited, so long as they express polypeptides *in vitro*, in *E. coli*, in cultured cells, or *in vivo*. However, preferred examples include the pBEST vector (ProMega) for *in vitro* expression, the pET vector (Invitrogen) for expression in *E. coli*, the pME18S-FL3 vector (GenBank Accession No. AB009864) for expression in cultured cells, and the pME18S vector (Mol. Cell Biol. 8:466-472(1988)) for *in vitro* expression, and such. A DNA of the present invention can be inserted into a vector by conventional methods, for example, by a ligase reaction using restriction enzyme sites (Current Protocols in Molecular Biology, edit. Ausubel, et al., (1987) Publish. John Wiley & Sons, Section 11.4-11.11).

Host cells to which the vectors of the present invention are introduced are not specifically limited, and various host cells can be used according to the objectives of the present invention. For example, bacterial cells (e.g. *Streptococcus*, *Staphylococcus*, *E. coli*, *Streptomyces*, *Bacillus subtilis*), fungal cells (e.g. yeast, *Aspergillus*), insect cells (e.g. Drosophila S2, Spodoptera SF9), animal cells (e.g. CHO, COS, HeLa, C127, 3T3, BHK, HEK293, Bowes melanoma cell), and plant cells are examples of cells for expressing polypeptides. The transfection of a vector to a host cell can be carried out by conventional methods, such as calcium phosphate precipitation methods, electroporation methods (Current protocols in Molecular Biology, edit., Ausubel et al., (1987) Publish. John Wiley & Sons, Section 9.1-9.9), Lipofectamine methods (GIBCO-BRL), microinjection methods, and such.

In host cells, appropriate secretion signals can be incorporated into a polypeptide of interest in order to facilitate the secretion of an expressed polypeptide into the lumen of the endoplasmic reticulum, into the cavity around a cell, or into the extracellular environment. These signals may be endogenous signals or signals from a species different to the target polypeptide.

When a polypeptide of the present invention is secreted into culture media, this culture media is collected to collect the polypeptide of the present invention. When a polypeptide of the present invention is produced intracellularly, the cells are first lysed, and the polypeptide is then collected.

In order to collect and purify a polypeptide of the present invention from a recombinant cell culture, methods known in the art can be used, including ammonium sulfate or ethanol precipitation, acid extraction, anionic or cationic exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography, and lectin chromatography.

<Antibodies>

The present invention provides antibodies that bind to a polypeptide of the present invention. Herein, the term "antibodies" refers to polyclonal antibodies, monoclonal antibodies, chimeric antibodies, single-stranded antibodies, humanized antibodies, and Fab fragments including Fab or other products of an immunoglobulin expression library.

A polypeptide of the present invention or its fragment, or analogs thereof, or a cell that expresses the same, can be used as an immunogen for producing antibodies that bind to a polypeptide of the present invention. The antibodies are preferably immunospecific to a polypeptide of the present invention. The term "immunospecific" means that an antibody has substantially higher affinity to polypeptides of the present invention compared to other polypeptides.

The antibodies binding to a polypeptide of the present invention can be prepared by methods known to those skilled in the art. For example, a polyclonal antibody can be obtained as follows: A polypeptide of the present invention, or a GST-fusion protein thereof, is administered to small animals, such as rabbits, to obtain serum. Polyclonal antibodies are prepared by purifying the serum by ammonium sulfate precipitation; a protein A or protein G column; DEAE ion exchange chromatography; an affinity column in which the polypeptide of the present invention is coupled; and such. On the other hand, monoclonal antibodies, for example, can be prepared as follows: A polypeptide of the present invention is administered to small animals such as mice, and their spleens are subsequently extirpated and ground down to separate the cells. The cells are then fused with mouse myeloma cells using reagents such as polyethylene glycol, and clones that produce antibodies binding to the polypeptide of the present invention are selected from these fused cells (hybridomas). The obtained hybridomas are then transplanted into mice peritoneal cavities, and ascites are collected from the mice. The monoclonal antibodies can be prepared by purifying the ascites using, for example, ammonium sulfate precipitation; a protein A or protein G column; DEAE ion exchange chromatography; an affinity column in which the polypeptides of the present invention are coupled; and such.

The antibodies of the present invention can also be used to detect and purify the polypeptides of the present invention in test samples.

<Detection of *Campylobacter* bacteria in test samples >

The present invention provides methods for detecting *Campylobacter* bacteria in test samples. Detecting *Campylobacter* bacteria in test samples is useful for various purposes, for example, in the diagnosis for campylobacteriosis, rapid examination of foods contaminated with *Campylobacter* bacteria, validation of food processing processes, and identification of causative bacteria at the time of food poisoning outbreak.

In one embodiment, the detection method of the present invention is a method for detecting the presence of *Campylobacter coli*, *Campylobacter jejuni*, and *Campylobacter fetus* in test samples, which includes the steps of:

(a) conducting a polymerase chain reaction on the test samples using a mixture of primer pairs specific to each of genomic DNAs encoding the cytolethal distending toxins of these bacteria; and

(b) determining the presence of these bacteria based on the presence or molecular weight of amplified fragments from the genomic DNAs encoding the cytolethal distending toxins of the bacteria.

In an alternative embodiment, the detection method of the present invention is a method for detecting the presence of *Campylobacter coli*, *Campylobacter jejuni*, and *Campylobacter fetus* in test samples, which includes the steps of:

(a) conducting a nucleic acid amplification reaction on test samples using a common primer pair that can amplify genomic DNAs encoding the cytolethal distending toxins of these bacteria;

(b) conducting a polymerase chain reaction using the genomic DNAs amplified in step (a) as a template and a mixture of primer pairs specific to each of genomic DNAs encoding the cytolethal distending toxins of the bacteria; and

(c) determining the presence of these bacteria based on the presence or molecular weight of amplified fragments from genomic DNAs encoding the cytolethal distending toxins of the bacteria.

PCR using multiple PCR primers in a single reaction system, as used in Examples, is called "multiplex PCR". Multiple bacterial species can be identified simultaneously by electrophoresing the PCR products and examining the sizes of their bands. The present invention provides methods for detecting *Campylobacter* bacteria by nucleic acid amplification methods, which include multiplex PCR as a typical example, using primers or combinations thereof that are suitably used to amplify multiple nucleic acid regions. There is no limitation on the type of nucleic acid amplification method in the present invention, as long as desired amplification products can be obtained. A preferred method is PCR.

Mixtures of specific primer pairs used in such methods include, for example, mixtures of the following primer pairs:

(a) a primer pair selected from SEQ ID NOs: 13, 14, and 28 to 36 to amplify genomic DNA encoding the cytolethal distending toxin of *Campylobacter coli*, or a primer pair that can amplify the same genomic DNA region as amplified with the primer pair;

(b) a primer pair selected from SEQ ID NOs: 11, 12, and 17 to 27 to amplify genomic DNA encoding the cytolethal distending toxin of *Campylobacter jejuni*, or a primer pair that can amplify the same genomic DNA region as amplified with the primer pair; and

(c) a primer pair selected from SEQ ID NOs: 15, 16, and 37 to 46 to amplify genomic DNA encoding the cytolethal distending toxin of *Campylobacter fetus*, or a primer pair that can amplify the same genomic DNA region as amplified with the primer pair. In addition, a primer pair selected from, for example, SEQ ID NOs: 7 to 10 and 47 to 50, or a primer pair that can amplify the same genomic DNA region as amplified with the primer pair, can be used as a common primer pair.

In a further embodiment of the present invention, the detection method is a method for detecting the presence of *Campylobacter coli*, *Campylobacter jejuni*, and *Campylobacter fetus* in test samples, which includes the steps of:

(a) conducting a nucleic acid amplification reaction on test samples using a common primer pair that can amplify genomic DNAs encoding the cytolethal distending toxins of these bacteria;

(b) digesting the genomic DNAs amplified in step (a) with a restriction enzyme; and

(c) determining the presence of these bacteria based on the molecular weight of DNA fragments resulting from the digestion. Restriction enzymes that can be used in this method are not particularly limited as long as it allows identification of genomic DNAs encoding the cytolethal distending toxins of *C. coli*, *C. jejuni*, and *C. fetus*, and include, for example, *Sau*3AI, *Dsa*I, *Mbo*I, *Rsa*I, *Eco*RI, *Hin*fI, *Nde*I, *Pst*I, *Xba*I, and *Xho*II. Meanwhile, examples of common primer pairs include primer pairs selected from SEQ ID NOs: 7 to 10 and 47 to 50, and primer pairs that can amplify the same genomic DNA region as amplified with the primer pairs.

A method for detecting polymorphisms based on lengths of fragments generated by digesting PCR-amplified DNA with various restriction enzymes, as described in the Example below, is called PCR-RFLP (PCR-Restriction Fragment Length Polymorphism). The present invention also provides primers that are suitably used in methods for detecting polymorphisms, which includes PCR-RFLP as a typical example, based on lengths of fragments generated by treating DNA amplified by nucleic acid amplification methods with various restriction enzymes.

In another embodiment of the present invention, the detection method is a method for detecting the presence of *Campylobacter* bacteria in test samples, which includes the steps of:

(a) conducting a nucleic acid amplification reaction on test samples using a common primer pair that can amplify genomic DNA encoding the cytolethal distending toxin of *Campylobacter* bacteria; and

(b) determining the presence of *Campylobacter* based on the presence or molecular weight of amplified fragments from genomic DNA encoding the cytolethal distending toxin of *Campylobacter* bacteria. Primer pairs used in this method are those that can amplify genomic DNAs encoding the cytolethal distending toxins of *Campylobacter* bacteria regardless of *Campylobacter* species. Such common primer pairs include, for example, primer pairs selected from SEQ ID NOs: 7 to 10, 47 to 50, and 64 to 67. As described above, the above primer pairs

are common primer pairs that amplify all of genomic DNAs encoding the cytolethal distending toxins of the three species, *Campylobacter coli*, *Campylobacter jejuni*, and *Campylobacter fetus*. The primer pairs described above are expected to amplify genomic DNAs encoding the cytolethal distending toxins of not only the above-described three species but also other *Campylobacter* bacteria. Likewise, primer pairs that can amplify the same genomic DNA region as amplified with the primer pairs described above may be amplify genomic regions of all the three bacterial species described above and other *Campylobacter* bacteria.

The present invention also provides kits used in the above detection methods of the present invention. These kits include an instruction manual in addition to the primer pairs described above. The kits may also include other components.

The detection of *Campylobacter* bacteria can be achieved at the protein level as well as at the DNA level as described above. The presence of these bacteria can be assessed in test samples by, for example, detecting the cytolethal distending toxins of the bacteria by Western blotting, dot blotting, immunoprecipitation, enzyme-linked immunosorbent assay (ELISA), immunofluorescence, or such using antibodies specific to the cytolethal distending toxins of the bacteria.

All prior art documents cited herein are incorporated herein by reference.

Examples

[Example 1] *Campylobacter* strains

C. jejuni, *C. coli*, and *C. fetus* collected from various clinical materials during 2001 to 2003 were used. Each strain was cultured in blood agar plates (Blood Agar Base No. 2: OXOID) containing 5% defibrinated horse blood (Japan Biological Material Center) and *Campylobacter* Selective Supplement SR69 (OXOID). *C. jejuni* and *C. coli* were cultured under 5% O_2, 10% CO_2, and 85% N_2 gases at 42°C, while *C. fetus* was cultured at 25°C in a low-temperature O_2/CO_2 gas incubator (MODEL9200: Wakenyaku Co., Ltd).

[Example 2] Preparation of PCR template

Five clones of each bacterial species were scraped off and suspended in 500 µl of sterile PBS. The harvested bacteria were washed by centrifugation at 10,000 rpm for 5 min (MRX-150: TOMY SEIKO Co., Ltd.), and then resuspended in 300 µl of sterile PBS. Then, the suspensions were boiled in boiling water bath for 10 minutes, and cooled on ice. The suspensions were centrifuged at 15,000 rpm for 10 min and the resulting supernatants were collected. The amount of DNA in the collected supernatants were quantified using a spectrophotometer (Ultrospec 3100pro: Amersham Biosciences). Each quantified cell extract

was diluted to 20 ng/μl and subjected to PCR.

[Example 3] Preparation of *C. coli* cdtB probe and Southern hybridization

A *C. coli* cdtB probe was prepared by PCR labeling using the primers GNW and LPF-D, DIG Labeling Mix (Roche), and cell extract of *C. coli* Co1-192 as a template.

Specifically, to test the existence of the *C. coli* CDT gene, three *C. jejuni* strains and two *C. coli* strains were analyzed by PCR using the degenerate primers GNW [SEQ ID NO: 5: 5'-GG(ACGT)AA(CT)TGGAT(ACT)TGGGG(ACGT)TA-3'] and LPF-D [SEQ ID NO: 6: 5'-(AGT)AA(CT)TG(ACGT)AC(AGT)TA(ACGT)CC(AGT)AA (ACGT)GG - 3'] described in a reference (Pickett, C. et al. Infect. Immun., 64: 2070 (1996)) under the condition of: 94°C for 3 minutes, 30 cycles of [94°C for 30 seconds, 42°C for 30 seconds, and 72°C for 2 minutes], and 72°C for 5 minutes. All three *C. jejuni* strains and two *C. coli* strains gave bands of the amplified cdt region at about 1.5 Kb (arrow 1 in Fig. 1).

The amplified bands were ligated into pT7 vector (Novagen), and *E. coli* (E.coli JM109) cells were transformed with the ligates. Sequencing of the resulting clones using a sequencer (ABI PRISM 377 DNA sequencer; Applied Biosystems) showed similar sequences to cdtB. BigDye terminator Cycle Sequencing Kits (Applied Biosystems) were used in the sequence reaction. In addition, 800 bp bands (arrow 2 in Fig. 1) were found to be cdtB-derived secondary bands, which were amplified because GNW primer was a mixed primer.

20 μg of *C. coli* Co1-192 genomic DNA was digested with 60 U of restriction enzyme *Hin*dIII at 37°C for 12 hours. Then, Southern blotting and DNA-DNA hybridization were performed using the prepared probe according to a conventional method (Molecular cloning: a laboratory manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, (2001)).

The hybridization was carried out under a stringent condition at 42°C. After blotting, the blot was washed twice with a 2x SSC/0.1% SDS solution at room temperature for five minutes, and then twice with a 0.2x SSC/0.1% SDS solution at 60°C for 15 minutes.

As a result, probe-positive bands were found at about 3 and 4 kbp (Fig. 2). The 3-kbp band was ligated into pUC18 vector. *E. coli* JM109 was transformed with the ligate, yielding a clone containing the cdtB region (3k44).

[Example 4] Sequencing of *C. coli* cdtB gene

The clone 3k44 containing the cdtB region of *C. coli* obtained in Example 3 was sequenced by a conventional method. The sequence of entire *C. coli* CDT region was determined as shown in SEQ ID NO: 1.

[Example 5] Design of common primer pair 1 and PCR

The *C. coli* CDT sequence of the present invention was compared with the CDT gene of *C. jejuni* from known databases to design common primers U and R described below. The primers were mixed and added to 1 μl of 20 ng/μl cell extract to give each primer concentration of 0.5 mM. The final volume was adjusted to 20 μl with PCR buffer (TaKaRa Ex Taq kit: Takara Bio). The reaction mixture was subjected to PCR under the condition of: 94°C for 3 minutes, 30 cycles of [94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute], and 72°C for 3 minutes. The result is shown in Fig. 3. Amplified fragments of about 1900 bp were found, and thus CDT-derived bands were detected from both *C. jejuni* (lanes 2 to 4) and *C. coli* (lanes 5 and 6).

Common primer U [SEQ ID NO: 7: GATAA(CT)GATCCTTTAAAACT]

Common primer R [SEQ ID NO: 8: (AT)(AT)CCAAAGCG(AT)TTTT(CG)TATGG]

[Example 6] Design of common primer pair 2 and PCR

Likewise, common primers Up and Do indicated below were designed, and PCR was carried out under the condition of: 94°C for 3 minutes, 30 cycles of [94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 45 seconds], and 72°C for 3 minutes. The results are shown in Figs. 4 to 6. Amplified fragments of about 720 bp were found.

Common primer Up [SEQ ID NO: 9: ACTTGGAATTTGCAAGGC]

Common primer Do [SEQ ID NO: 10: TCTAAAATTTAC(ACT)GGAAAATG]

[Example 7] Design of specific primers and detection of cdtB gene by multiplex PCR

The *C. coli* CDT sequence of the present invention was compared with the CDT gene of *C. jejuni* from known databases to design *C. jejuni*-specific primers CjSPBU3 and CjSPBR3 described below. Likewise, *C. coli*-specific primers CcSPBU5 and CcSPBR5, and *C. fetus*-specific primers CfSPBU1 and CfSPBR1 were designed.

The primers were combined and added to 1 μl of 20 ng/μl cell extract to give each primer concentration of 0.5 mM. The final volume was adjusted to 20 μl with PCR buffer (TaKaRa Ex Taq kit: Takara Bio). The reaction mixture was subjected to multiplex PCR under the condition of: 94°C for 3 minutes, 30 cycles of [94°C for 56 seconds, 55°C for 30 seconds, and 72°C for 45 seconds], and 72°C for 3 minutes (GeneAmp PCR system 9700; Applied Biosystems). The result is shown in Fig. 7. Amplified CDT fragments specific to *C. jejuni* (about 750 bp), *C. coli* (about 400 bp), and C. fetus (about 530 bp) were found, allowing the discrimination of *C. jejuni* (lanes 2 to 4), *C. coli* (lanes 5 and 6), and *C. fetus* (lanes 7 and 8).

Specific primer CjSPBU3 [SEQ ID NO: 11: TACTCCGCCTTTTACCGCA]

Specific primer CjSPBR3 [SEQ ID NO: 12: GAGTATAGGTTTGTTGTC]

Specific primer CcSPBU5 [SEQ ID NO: 13: TTTAATGTATTATTTGCCGC]

Specific primer CcSPBR5 [SEQ ID NO: 14: TCATTGCCTATGCGTATG]
Specific primer CfSPBU1 [SEQ ID NO: 15: CGCAAGTTGGAAGACTAT]
Specific primer CfSPBR1 [SEQ ID NO: 16:TTTATTATCGCCGGAGCA]

[Example 8] Identification of bacterial species by PCR-RFLP using common primer pair 1

After PCR using common primer pair 1 obtained in Example 6, 5U of the restriction enzyme *Sau*3AI (NEB) was added to 8.5 μl of the reaction solution. The resulting mixture was reacted at 37°C for 3 hours and then electrophoresed. The result is shown in Fig. 8.

[Example 9] Detection of cdtB gene by multiplex PCR using specific primers

Multiplex PCR was performed on other various clinical strains of *Campylobacter* bacteria by using specific primers obtained in Example 7 and the experimental condition in Example 7. The result is shown in Fig. 9. As in the case of Example 7, amplified CDT fragments specific to *C. jejuni* (about 750 bp), *C. coli* (about 400 bp), and *C. fetus* (about 530 bp) were found, allowing the discrimination of each species.

[Example 10] Preparation of *C. fetus* cdtB probe and Southern hybridization

A *C. fetus* cdtB probe was prepared by PCR labelling using common primer pair 2 (common primers Up and Do), DIG Labeling Mix (Roche), and cell extract of *C. fetus* Co1-187 as a template.

20 μg of genomic DNA of *C. fetus* Co1-187 was digested with 60 U of the restriction enzyme *Hin*dIII at 37°C for 12 hours. Then, Southern blotting and DNA-DNA hybridization were carried out according to a conventional method (Molecular cloning: a laboratory manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, (2001)) using the obtained probe. The hybridization was carried out under a stringent condition at 42°C. After blotting, the blot was washed twice with a 2x SSC/0.1% SDS solution at room temperature for five minutes, and then twice with a 0.2x SSC/0.1% SDS solution at 60°C for 15 minutes.

As a result, probe-positive bands were found at about 2 and 5 kbp (Fig. 10). The 2-kbp bands was ligated into pUC18 vector. *E. coli* JM109 was transformed with the ligate, yielding a clone containing the cdtB region (Cf78).

[Example 11] Sequencing of *C. fetus* CDT gene

The clone Cf78 containing the cdtB region of *C. fetus* obtained in Example 10 was sequenced by a conventional method to determine the sequences of the cdtA and cdtB regions of *C. fetus*. Since the clone Cf78 did not contain cdtC region, the sequence of cdtC region was determined by performing gene walking under the condition described below using random

primers designed based on the determined cdtB gene sequence. Thus, the sequence of the entire *C. fetus* CDT region was determined as shown in SEQ ID NO: 51.

[Gene walking using random primer]

The primer sets consisting of a random primer, target amplification primer, and sequencing primer described below were designed based on the gene sequence determined in Example 11. The targets were amplified using the *C. fetus* Col-187 gene as a template. For the target amplification, 10 pmol of the random primer was added to 20 ng of the template gene, and the final volume was adjusted to 100 µl using KOD Dash PCR Kit (TOYOBO). The reaction mixture was subjected to PCR under the condition of: 94°C for 2 minutes and 35 cycles of [94°C for 20 seconds, 65°C for 5 seconds, and 74°C for 30 seconds].

The resulting PCR products were sequenced according to a conventional method using the sequencing primers.

Primer set 1

Random primer [SEQ ID NO: 55: GCTTGTAGCAGTATTGATGCNNNNNNNNN]

Target amplification primer [SEQ ID NO: 56: GCTTGTAGCAGTATTGATGC]

Sequencing primer [SEQ ID NO: 57: CTAGTTTCGGACCATTTTCC]

Primer set 2

Random primer [SEQ ID NO: 58: ATACGCAATGCAAACACCGGNNNNNNNNN]

Target amplification primer [SEQ ID NO: 59: ATACGCAATGAAACACCGG]

Sequencing primer [SEQ ID NO: 60: TAAAAGCGATTTTCAGGGCAG]

Primer set 3

Random primer [SEQ ID NO: 61: TGTCGACATAGAGCCTAAACNNNNNNNNN]

Target amplification primer [SEQ ID NO: 62: TGTCGACATAGAGCCTAAAC]

Sequencing primer [SEQ ID NO: 63: ATTTTCACCGCCGCTTAGTG]

[Example 12] Design of cdtA common primers and PCR

The cdtA sequences of *C. coli* and *C. fetus* of the present invention were compared with the cdtA gene of *C. jejuni* from known databases to design cdtA common primers U and R described below. The primers were combined and added to 1 µl of 20 ng/µl cell extract to give each primer concentration of 0.25 mM. The final volume was adjusted to 20 µl with PCR buffer (TaKaRa Ex Taq kit: Takara Bio). The reaction mixture was subjected to PCR under the condition of: 94°C for 3 minutes, 30 cycles of [94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds], and 72°C for 3 minutes. The result is shown in Fig. 11 (left). Amplified fragments of about 550 bp were found. Thus, cdtA-derived bands were detected for

all of *C. jejuni* (lanes 2 to 4), *C. coli* (lanes 5 and 6), and *C. fetus* (lanes 7 and 8).

CdtA common primer U
[SEQ ID NO: 64: (GA)A(ACT)GAT(AC)(AC)(TAG)GAT(AC)GATCC(AT)(TC)CAAA]
CdtA common primer R
[SEQ ID NO: 65: (GA)(AT)AA(TC)AGG(TC)G(CT)TTG(CT)A(AT)(GA)CA]

[Example 13] Detection of cdtA gene by PCR using cdtA common primers

PCR was performed on other various clinical strains of *Campylobacter* bacteria using the common primers obtained in Example 12 and the experimental condition in Example 12. The result is shown in Fig. 12. As in the case of Example 12, amplified fragments specific to cdtA (about 550 bp) were found.

[Example 14] Design of cdtC common primers and PCR

The cdtC sequences of *C. coli* and *C. fetus* of the present invention were compared with the cdtC gene of *C. jejuni* from the known database (BLAST) to design cdtC common primers U and R described below.

1 μl of 20 ng/μl cell extract and the primers were combined to give each primer concentration of 0.25 mM. The final volume was adjusted to 20 μl with PCR buffer (TaKaRa Ex Taq kit: Takara Bio). The reaction mixture was subjected to PCR under the condition of: 94°C for 3 minutes, 30 cycles of [94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 minutes], and 72°C for 3 minutes. The result is shown in Fig. 3. Amplified fragments of about 320 bp were found. Thus, cdtC-derived bands were detected for all of *C. jejuni* (lanes 10-12), *C. coli* (lanes 13 and 14), and *C. fetus* (lanes 15 and 16) (Fig. 11; right).

CdtC common primer U
[SEQ ID NO: 66:(AGC)A(TG)(TC)(TC)(AT)(AG)(AT)(AT)(GT)A(CT)CAAAA(CT)TGG]
CdtC common primer R
[SEQ ID NO: 67:(AGC)CTA(AGT)(AT)CC(AT)A(AC)(GT)C(GT)(AT)T(CT)TT(GC)]

[Example 15] Detection of cdtC gene by PCR using cdtC common primers

PCR was performed on other various clinical strains of *Campylobacter* bacteria using the common primers obtained in the Examples and the experimental condition in Example 14. The result is shown in Fig. 13. As in the case of Example 14, amplified fragments specific to cdtC (about 320 bp) were found.

[Example 16] Design of cdtA specific primers and detection of cdtA gene by multiplex PCR

The CDT sequence of *C. fetus* of the present invention was compared with the CDT genes of *C. jejuni* and *C. coli* from the known database (BLAST) to design *C. jejuni*-specific primers CjASPU2 and CjASPR2 described below. Likewise, the *C. coli*-specific primers CcASPU1 and CcASPR1 and *C. fetus*-specific primers CfASPU1 and CfASPR1 were designed.

The primers were combined and added to 1 μl of 20 ng/μl cell extract to give each primer concentration of 0.125 mM. The final volume was adjusted to 20 μl with PCR buffer (TaKaRa Ex Taq kit: Takara Bio). The reaction mixture was subjected to multiplex PCR under the condition of: 94°C for 3 minutes, 30 cycles of [94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds], and 72°C for 3 minutes (GeneAmp PCR System 9700; Applied Biosystems). The result is shown in Fig. 14 (left). Amplified CDT fragments specific to *C. jejuni* (about 630 bp), *C. coli* (about 330 bp), and *C. fetus* (about 490 bp) were found, allowing the discrimination of *C. jejuni* (lanes 2 to 4), *C. coli* (lanes 5 and 6), and *C. fetus* (lanes 7 and 8).

Specific primer CjASPU2 [SEQ ID NO: 68: AGGACTTGAACCTACTTTTC]
Specific primer CjASPR2 [SEQ ID NO: 69: AGGTGGAGTAGTTAAAAACC]
Specific primer CcASPU1 [SEQ ID NO: 70: ATTGCCAAGGCTAAAATCTC]
Specific primer CcASPR1 [SEQ ID NO: 71: GATAAAGTCTAAAACTGC]
Specific primer CfASPU1 [SEQ ID NO: 72: AACGACAAATGTAAGCACTC]
Specific primer CfASPR1 [SEQ ID NO: 73:TATTTATGCAAGTCGTGCGA]

[Example 17] Detection of cdtA gene by multiplex PCR using cdtA-specific primers

Multiplex PCR was performed on other various clinical strains of *Campylobacter* bacteria using the specific primers obtained in the Examples and the experimental condition in Example 14. The result is shown in Fig. 15. As in the case of Example 14, amplified cdtAs fragments specific to *C. jejuni* (about 630 bp), *C. coli* (about 330 bp), and *C. fetus* (about 490 bp) were found, allowing the discrimination of each species.

[Example 18] Design of cdtC specific primers and detection of cdtC gene by multiplex PCR

The CDT sequence of *C. fetus* of the present invention was compared with the CDT genes of *C. jejuni* and *C. coli* from known databases to design *C. jejuni*-specific primers CjCSPU1 and CjCSPR2 described below. Likewise, *C. coli*-specific primers CcCSPU1 and CcCSPR1, and *C. fetus*-specific primers CfCSPU2 and CfCSPR1 were designed.

The primers were combined and added to 1 μl of 20 ng/μl cell extract to give each primer concentration of 0.125 mM. The final volume was adjusted to 20 μl with PCR buffer (TaKaRa Ex Taq kit: Takara Bio). The reaction mixture was subjected to multiplex PCR under

the condition of: 94°C for 3 minutes, 30 cycles of [94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds], and 72°C for 3 minutes (GeneAmp PCR System 9700; Applied Biosystems). The result is shown in Fig. 14 (right). Amplified CDT fragments specific to *C. jejuni* (about 500 bp), *C. coli* (about 300 bp), and *C. fetus* (about 400 bp) were found, allowing the discrimination of *C. jejuni* (lanes 10 to 12), *C. coli* (lanes 13 and 14), and *C. fetus* (lanes 15 and 16).

Specific primer CjCSPU1 [SEQ ID NO: 74: TTTAGCCTTTGCAACTCCTA]
Specific primer CjCSPR2 [SEQ ID NO: 75: AAGGGGTAGCAGCTGTTAA]
Specific primer CcCSPU1 [SEQ ID NO: 76: TAGGGGATATGCACGCAAAAG]
Specific primer CcCSPR1 [SEQ ID NO: 77: GCTTAATACAGTTACGATAG]
Specific primer CfCSPU2 [SEQ ID NO: 78: AAGCATAAGTTTTGCAAACG]
Specific primer CfCSPR1 [SEQ ID NO: 79: GTTTGGATTTTCAAATGTTCC]

[Example 19] Detection of cdtC gene by multiplex PCR using specific primers

Multiplex PCR was performed on other various clinical strains of *Campylobacter* bacteria using the specific primers obtained in the Examples and the experimental condition in Example 14. The result is shown in Fig. 16. As in the case of Example 14, amplified CdtC fragments specific to *C. jejuni* (about 500 bp), *C. coli* (about 300 bp), and *C. fetus* (about 400 bp) were found, allowing the discrimination of each species.

Industrial Applicability

The primers of the present invention are applicable not only to epidemiologic studies and researches on *Campylobacter* bacteria and diagnosis of campylobacteriosis but also to the rapid examination of foods contaminated with *Campylobacter* bacteria, validation of food processing processes, and rapid identification of the causative bacteria at the time of food poisoning outbreak, and therefore useful in preventing expansion of infection.